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2. Patent application number
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9903851.5
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*
STUART HARBRON
44 SWING GATE LANE
BERKHAMSTED
HERTFORDSHIRE HP4 2LL

Patents ADP number *(if you know it)*
04184487001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention
METHOD FOR DETERMINING NUCLEASE ACTIVITY

5. Name of your agent *(if you have one)*
Sommerville & Rushton
45 Grosvenor Road
St. Albans
Herts. AL1 3AW

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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1151001

	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
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	Number of earlier application	Date of filing *(day / month / year)*
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		

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11. I/We request the grant of a patent on the basis of this application.

Signature S Harbron Date 19/2/99

12. Name and daytime telephone number of person to contact in the United Kingdom STUART HARBRON 01442 385740.

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Method for determining nuclease activity.

The present invention is concerned with a method for detecting the presence of a nuclease enzyme.

Self (EP0027036A, EP0049606A, EP0058539A, EP0060123A, US4446231, US4595655, US4598042, and US4769321) discloses methods for detecting phosphatase enzymes that produce NAD or NADH from NADP or NADPH respectively.

Akihiro (US5589349) discloses the use of enzymes with improved stability in a cycling assay for alkaline phosphatase.

Fisher et al disclose the assay of nucleases using FADP as a substrate (WO98/19168A).

Harbron et al (Analytical Biochemistry (1991) **198**:47-51) disclose an assay for alkaline phosphatase which relies on the production of FMN, which is detected using apoglycolate oxidase.

Harbron et al (Journal of Bioluminescence and Chemiluminescence (1991) **6**:251-258) disclose the luminometric detection of alkaline phosphatase based on the production of FMN, which is detected using the bacterial bioluminescent system.

Harbron (GB2324370B) discloses the use of nuclease P1 in a nucleic acid hybridisation assay in which excess probe is destroyed.

Stanley (Methods in Enzymology (1978) **57**:215-223) discloses the quantitation of NADH, NADPH and FMN using bacterial luciferase.

Rabin et al. (US4745054) discloses prosthetogenic enzyme amplification assays in which a pyrimidine ribonucleoside 3'-phosphate ester RpX is hydrolysed by ribonuclease to give XOH. XOH is a prosthetic group or a prosthetic group precursor such as thiamine, riboflavin, pyridoxal or pyridoxamine.

A number of patents assigned to Tropix describe 1,2-dioxetane derivatives of utility in chemiluminometric detection (US5869705, US5869699, US5866389, US5856522, US5851771, US5847161, US5840919, US5783381, US5777133, US5763681, US5756770, US5707559, US5679803, US5679802, US5652345, US5639907, US5637747, US5625077, US5605795, US5538847, US5397852, US5342966, US5330900, US5326882, US5225584, US5220005, US4978614, US4956477, US4931569, US4931223, US5843681, US5831102, US5773628, US5591591, US5582980, US5543295, US5145772, and US4952707).

The above citations are included herein by reference in their entirety.

Broadly, the present invention discloses a method for detecting a nuclease enzyme which comprises contacting said nuclease enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety, whereby ROH and pX are produced, and detecting said ROH or said pX moiety.

In a further aspect, the invention provides a method for detecting a nuclease enzyme that is free in solution, immobilised on a surface, or attached to a member of a specific binding pair. The method of the invention may thus be applied as a detection step in nucleic acid hybridisation assays, enzyme immunoassays and ligand:receptor binding assays.

In a further aspect the invention provides a variety of methods for detecting the detectable moieties produced. These include fluorometric, colorimetric, and luminometric endpoints. Enzyme cycling and apoenzyme reactivation assays are also provided.

In a further aspect the invention provides a kit for carrying out the method.

Preferred embodiments of the invention may enable one to achieve one or more of the following objects and advantages:

(a) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields products that are easily detected. An advantage of the present invention is that the assay may be easily performed using equipment commonly available in the laboratory.

(b) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that is a prosthetic groups for an enzyme. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(c) to provide a method for the detection of a nuclease enzyme that utilises a substrate which yields a product that can be detected by chemiluminescent or bioluminescent means. An advantage of the present invention is that the assay is rapid and/or has high sensitivity.

(d) to provide a method to detect a complex formed between two members of a specific binding pair, in which one of said members is labelled with a nuclease enzyme. An advantage of the present invention is that the complex may be rapidly and/or sensitively detected.

(c) to provide a kit for carrying out the method of the invention.

Some embodiments of the invention will be described in more detail, by way of example, with reference to the accompanying drawings in which:

Figure 1 is a diagrammatic representation of the conversion of NAD3P to NAD through the action of a nuclease enzyme, and subsequent

cycling of the NAD produced through the action of a dehyrogenase and a diaphorase enzyme, to produce a coloured formazan.

Figure 2 is a diagrammatic representation of the hydrolysis of a substrate by a nuclease enzyme to yield products for detection.

Figure 3 is a diagrammatic representation of the hydrolysis of a adenosine-3'-phosphoriboflavin derivative by the action of a nuclease enzyme to yield FMN, and subsequent reconstitution of an apoenzyme by the FMN to yield holoenzyme for detection.

Figure 4 is a diagrammatic representation of the hydrolysis of a nucleoside-3'-phospho-1,2-dioxetane derivative to yield the corresponding 1,2-dioxetane phosphate. This latter is converted to 1,2-dioxetane, which decomposes producing light.

Figure 5 is a standard curve for the detection of nuclease P1 in a NAD-NADH cycling reaction. The absorbance produced after 800 sec at different pH values is plotted against the amount of nuclease P1 present in the reaction mixture.

Figure 6 is a standard curve for the detection of FMN in an apoenzyme reconstitution assay. The absorbance produced at 324 nm is plotted against the concentration of FMN in an aliquot added to the reaction mixture for two different apoenzyme preparations: circle is sugar beet, triangle is spinach.

The present invention provides a method for detecting a nuclease enzyme.

The method comprises contacting a nuclease enzyme with a compound of formula RpX, wherein R is a 3'nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety. The nuclease enzyme cleaves this compound at the 3'phosphate group to

yield ROH and pX. Substituent R and X are chosen so that either ROH or pX may be easily detected.

The present invention provides a variety of methods for detecting ROH or pX. These approaches may be colorimetric, fluorimetric, or luminometric, or may be through enzyme cycling reactions or apoenzyme reactivation assays.

In a preferred embodiment, the substrate RpX is NAD3′phosphate (NAD3P). This differs from commonly occurring NADP, which carries the phosphate moiety at the 2' position. This is hydrolysed by a nuclease to give NAD, which may be easily and sensitively detected, either by spectrophotometry or fluorimetry, or by coupling with a bacterial luminescence system to produce light, as disclosed by Stanley, or through enzyme cycling, as disclosed by Self. In a preferred embodiment, NAD is converted to NADH through the action of a dehydrogenase enzyme. The dehydrogenase enzyme may be alcohol dehydrogenase or lactate dehydrogenase. The presence of NADH may be detected spectrophotometrically or fluorometrically. Referring to Fig. 1, which shows a particularly preferred embodiment, NAD3P is hydrolysed to give NAD. NAD is converted to NADH through the action of a dehydrogenase enzyme. A diaphorase enzyme reduces a tetrazolium compound, such as INT, to give NAD and a coloured formazan, the absorption of which can be measured at 492nm. The NAD produced may then be cycled back to NADH through the action of the dehydrogenase enzyme, leading to an ever-increasing rate of colour development. A similar approach may be used using NAD3PH as the substrate, which yields NADH on hydrolysis. In this embodiment, the NADH enters the cycle as a substrate for diaphorase.

Referring now to Fig. 2, which shows another preferred embodiment, the substrate is a nucleosidyl-3′-phosphodiester wherein

X is, for example, riboflavin, thiamine, pyridoxamine or pyridoxal, B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. These are hydrolysed by the nuclease enzyme to yield, for example, riboflavin phosphate (FMN), thiamine phosphate, pyridoxamine phosphate or pyridoxal phosphate, respectively. These may be detected using an apoenzyme reactivation assay of the type disclosed by Rabin. For example, FMN may be detected using apoglycolate oxidase; pyridoxal phosphate may be detected using apoaminoacid transferase. In a particularly preferred embodiment, shown in Fig. 3, the substrate adenosine-3'-phosphoriboflavin wherein A is adenine and R' is H. This compound is hydrolysed by the nuclease enzyme to yield adenosine and FMN (riboflavin phosphate). FMN may be sensitively detected using an apoenzyme, such as apoglycolate oxidase, as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51), or by bioluminescent detection, as described by Harbron et al. (Journal of Bioluminescence and Chemiluminescence (1991) **6**:251-258). In an analogous fashion, RpX may be adenosine-3'-phosphothiamine, adenosine-3'-phosphopyridoxamine or adenosine-3'-phosphopyridoxal, which upon hydrolysis yield thiamine phosphate, pyridoxamine phosphate or pyridoxal phosphate. These two may be sensitively detected using the corresponding apoenzyme. Apoenzymes: glycolate oxidase from spinach, transaminases.

In a further preferred embodiment, illustrated in Figure 4, the substrate is a nucleosidyl-3´-phospho-1,2-dioxetane derivative, such as a nucleosidyl-3´-phosphoadamantyl derivative, wherein B is a base, for example adenine, guanine, uracil, thymine or cytosine, or a derivative thereof, and R' is H or other substituent. This is hydrolysed to yield an adamantyl phosphate derivative, which may be hydrolysed chemically or in the presence of a phosphatase enzyme to

yield the corresponding adamantyl derivative, which decomposes chemiluminometrically. A further preferred embodiment utilises an adenosine-3'-phosphoadamantyl derivative, which, upon hydrolysis, yields an adamantyl-phosphate derivative. This may then be dephosphorylated by means of a phosphatase enzyme or chemically. For example, the adamantyl-phosphate derivative produced may be CDP-*Star* (R) from Tropix Inc. Upon dephosphorylation of CDP-*Star* (R) substrate by alkaline phosphatase, a metastable chlorophenolate dioxetane anion intermediate is formed which decomposes and emits light at a maximum wavelength of 466 nm. A delay in reaching maximum light emission results since the dioxetane anion has a half-life of less than one minute to several hours, depending on the surrounding environment. Film or simple instrumentation may be used to quantitate the chemiluminescent signal, which is produced as a continuous glow due to the reaction kinetics of the system.

The nuclease enzyme is any enzyme that cleaves the substrate RpX to yield R and pX. In one embodiment the nuclease enzyme is an enzyme of class EC.3.1.30.1. In a preferred embodiment the nuclease enzyme is nuclease P1, nuclease S1 or mung bean nuclease. In a particularly preferred embodiment the nuclease enzyme is nuclease P1.

In one embodiment the nuclease enzyme is free in solution. In another embodiment the nuclease enzyme is immobilised on a surface. In further embodiments the nuclease enzyme is attached to one member of a specific binding pair.

The present invention provides a method for detecting binding events between specific binding pairs, in which one of the pair is labelled with the nuclease enzyme. The covalent attachment of the nuclease enzyme to this moiety is described in Fisher et al.

(WO98/19168) and Harbron (GB2324370B), and may be achieved by a number of well-known methods using a wide range of heterobifunctional reagents. For example, the method of Carlsson *et al.* (*Biochem J* (1978) **173**: 723 - 737) may be used: the nuclease enzyme is reacted with 3-[(2)-pyridyldithio]propionic acid N-hydroxysuccinimide ester (SPDP) to give a 2-pyridyl disulphide-activated label. This allows disulphide exchange with a specific binding partner having a sulphydryl group to yield a labelled specific binding partner. Other approaches for labelling the specific binding partner will be apparent to one skilled in the art.

In one embodiment the specific binding pair comprises an antibody and a hapten or antigen. In another embodiment the specific binding pair comprises a nucleic acid probe and its corresponding target sequence. In a further embodiment the specific binding pair comprises a biotin derivative and avidin, streptavidin or neutravidin. In a yet further embodiment the specific binding pair comprises a ligand and a receptor.

Thus the invention may be used to detect binding events in nucleic acid hybridisation assays, enzyme immunoassays, and receptor:ligand binding assays.

The present invention provides a kit for carrying out the method of the invention. The kit comprises a compound of formula RpX, and a detection system for detecting ROH or pX. In one embodiment, RpX is NAD3P

The following examples illustrate aspects of the invention, and are not intended to limit the scope of the invention.

EXAMPLE 1 - Assay of Nuclease P1

A premix containing the following reagents was prepared prior to the assay and stored at 4°C until required: 50µl 0.5 M citrate buffer, pH 6.3; 100µl 10 mM INT; 10µl 5 mM NAD3´P; 10µl ethanol; 30µl diaphorase solution (30 U/ml); 10µl alcohol dehydrogenase solution (3mg/ml) and 780 µl of water.

10 µl aliqouts from a serially diluted solution of nuclease P1 were dispensed into the wells of a microtitre plate. 90µl of the premix were then added, and the plate incubated at room temperature. The change in absorbance at 490 nm was followed by means of a plate reader.

The performance of the assay is illustrated in Figure 5, which shows a standard curve for the assay of nuclease P1 using the above assay at pH 6.3, and at pH 6.0 and 6.7.

EXAMPLE 2 - Apoenzyme Reactivation Assay for the Detection of FMN

Apoglycolate oxidase was prepared as described by Harbron et al. (Analytical Biochemistry (1991) **198**:47-51). A standard curve for the estimation of FMN, shown in Fig. 6, was prepared as follows: 50 mM Tris-HCl buffer, pH8.3, 44 mU apoglycolate oxidase and 0.02 to 200µM FMN in a total volume of 0.05 mL was incubated for 1h at room temperature. This was then added to 0.95 mL of 50 mM Tris-HCl buffer, pH8.3, containing 3.47 mM phenylhydrazine and 5.26 mM glycolic acid, and the linear rate of absorbance was measured at 324 nm.

Claims

I claim:

1. A method for detecting a nuclease enzyme comprising the steps:
   (a) contacting said enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety, whereby ROH and pX are produced, and
   (b) detecting said ROH or said pX moiety
2. A method for detecting a complex formed between two members of a specific binding pair, in which one of said members is labelled with a nuclease enzyme, comprising the steps:
   (a) contacting said enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety, whereby ROH and pX are produced, and
   (b) detecting said ROH or said pX moiety
3. The method of claim 2 in which said specific binding pair comprises an antibody and an antigen.
4. The method of claim 2 in which said specific binding pair comprises an antibody and a hapten.
5. The method of claim 2 in which said specific binding pair comprises a probe nucleic acid and a target nucleic acid sequence.
6. The method of claim 2 in which said specific binding pair comprises biotin or a biotin derivative and avidin, streptavidin or neutravidin.
7. The method of claim 2 in which said specific binding pair comprises a receptor and a ligand.
8. The method of claims 1 to 7 wherein ROH is NAD and X is H.

9. The method of claims 1 to 7 wherein ROH is NADH and X is H.

10. The method of claims 1 to 7 wherein X is riboflavin or a riboflavin derivative, and pX is FMN or a FMN derivative.

11. The method of claims 1 to 7 wherein X is pyridoxal or a pyridoxal derivative, and pX is pyridoxal phosphate or a pyridoxal phosphate derivative.

12. The method of claims 1 to 7 wherein X is pyridoxamine or a pyridoxamine derivative, and pX is pyridoxamine phosphate or a pyridoxamine phosphate derivative.

13. The method of claims 1 to 7 wherein X is thiamine or a thiamine derivative, and pX is thiamine phosphate or a thiamine phosphate derivative.

14. The method of claims 1 to 7 wherein X is a 1,2-dioxetane derivative, and pX is a 1,2-dioxetane-phosphate derivative

15. The method of claim 8 or 9 wherein said detecting step comprises conducting enzymatic cycling of NAD-NADH interconversions in the presence of a dehydrogenase, a substrate for said dehydrogenase, a terazolium dye and a diaphorase, and detecting the amount of the NAD or NADH with a colour-development signal of formazan which is produced by the action of diaphorase and NADH-NAD conversions.

16. The method of claims 1 or 7 wherein said detecting step comprises contacting said pX with an apoenzyme.

17. The method of claim 10 wherein said detecting step comprises contacting said FMN or FMN derivative with an apoenzyme.

18. The method of claim 17 wherein said apoenzyme is apoglycolate oxidase.

19. The method of claim 11 wherein said detecting step comprises contacting said pyridoxal phosphate or pyridoxal phosphate derivative with an apoenzyme.

20. The method of claim 12 wherein said detecting step comprises contacting said pyridoxamine phosphate or pyridoxamine phosphate derivative with an apoenzyme.

21. The method of claim 19 or 20 wherein said apoenzyme is an apotransaminase.

22. The method of claim 13 wherein said detecting step comprises converting said thiamine phosphate or thiamine phosphate derivative to thiamine pyrophosphate or a thiamine pyrophosphate derivative and contacting said thiamine pyrophosphate or thiamine pyrophosphate derivative with an apoenzyme.

23. A kit for detecting the presence of a nuclease enzyme comprising:

(a) a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety, and

(b) a detection system for detecting ROH or pX.

22. The kit of claim 21 wherein RpX is NAD3P or NAD3PH.

23. The kit of claim 21 wherein RpX is a nucleoside-3'phosphoriboflavin derivative.

24. The kit of claim 21 wherein RpX is a nucleoside-3'-phospho-pyridoxal derivative.

25. The kit of claim 21 wherein RpX is a nucleoside-3'-phospho-pyridoxamine derivative.

26. The kit of claim 21 wherein RpX is a nucleoside-3'-phospho-thiamine derivative.

27. The kit of claim 21 wherein RpX is a nucleoside-3'-phospho-1,2-dioxetane derivative.

28. The kit of claim 21 wherein said detection system comprises a dehydrogenase, a diaphorase, and a tetrazolium compound.

29. The kit of claim 21 wherein said detection system comprises an apoenzyme.

30. The kit of claim 21 wherein said detection system comprises a phosphatase.

Abstract

A method for detecting a nuclease enzyme is disclosed which comprises contacting the enzyme with a compound of formula RpX, wherein R is a 3´nucleosidyl derivative, p is a phospho radical, and X is H or any esterifiable moiety, whereby ROH and pX are produced, and detecting ROH or pX.

In a further aspect, the invention provides a method for detecting a nuclease enzyme that is free in solution, immobilised on a surface, or attached to a member of a specific binding pair. The method of the invention may thus be applied as a detection step in nucleic acid hybridisation assays, enzyme immunoassays and ligand:receptor binding assays.

In a further aspect the invention provides a variety of methods for detecting the detectable moieties produced. These include fluorometric, colorimetric, and luminometric endpoints. Enzyme cycling and apoenzyme reactivation assays are also provided.

Figure 1

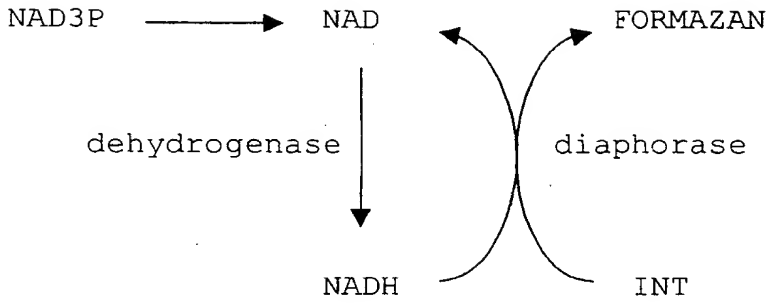


Figure 2

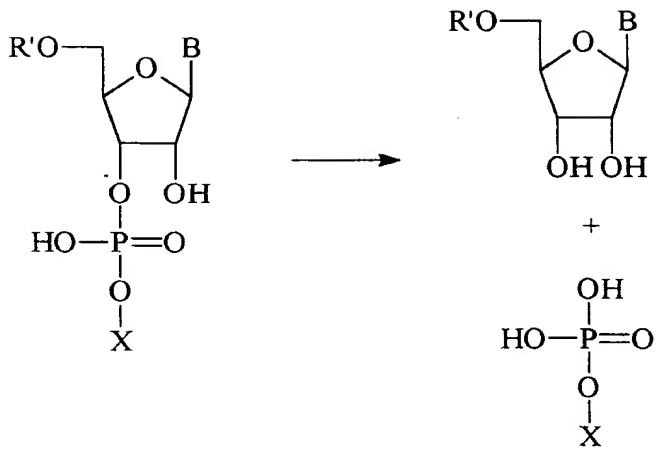


Figure 3

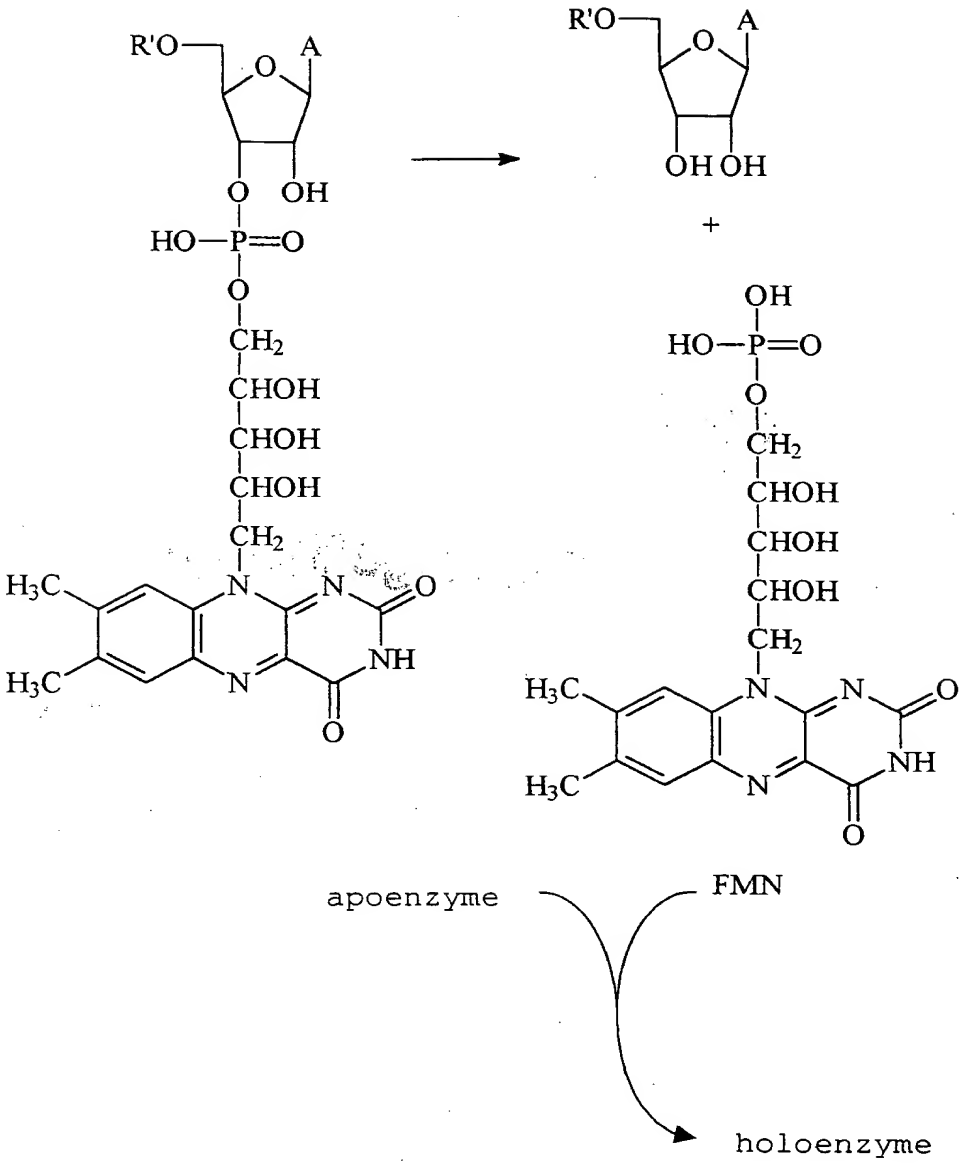

R'O
A
O
OH
HO—P=O
CH2
CHOH
CHOH
CHOH
CH2
H3C
H3C
N
N
O
NH
+
apoenzyme
FMN
holoenzyme

Figure 4

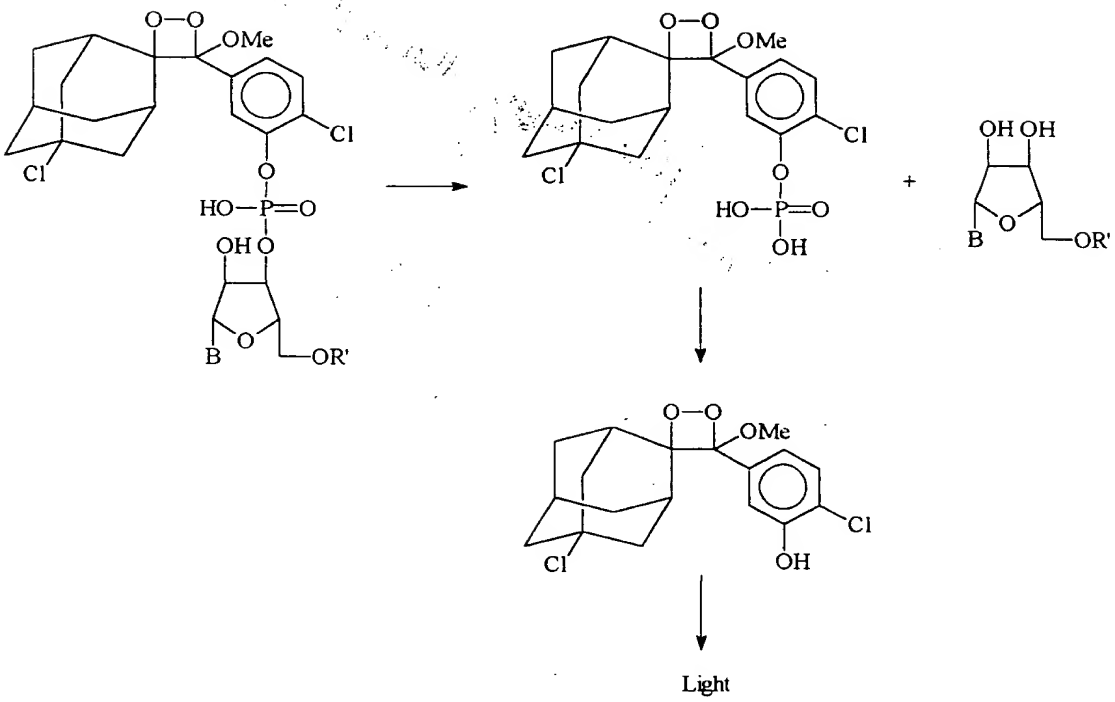


Figure 5

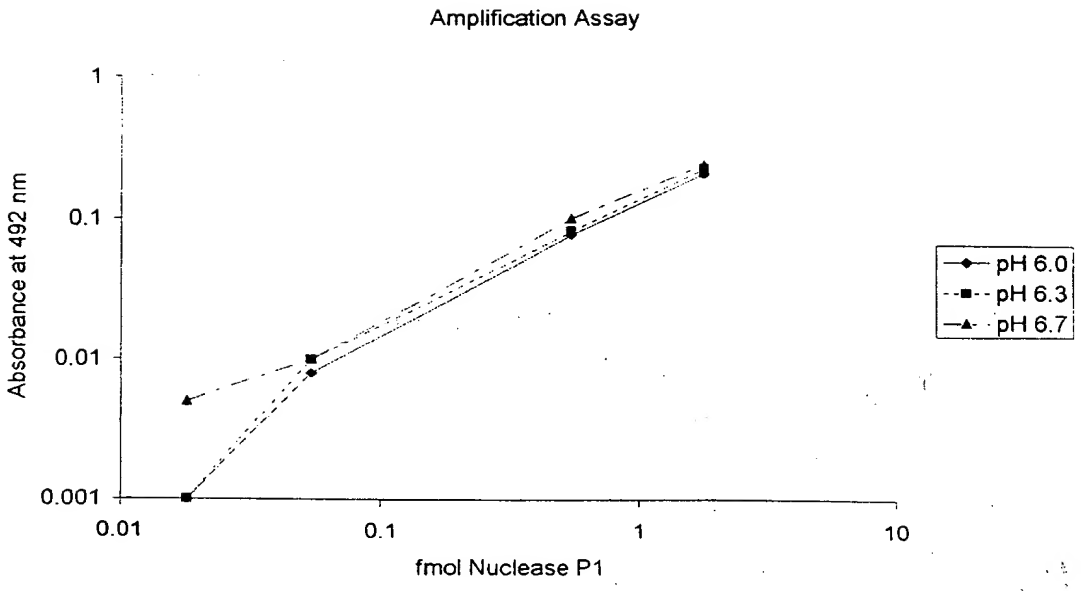
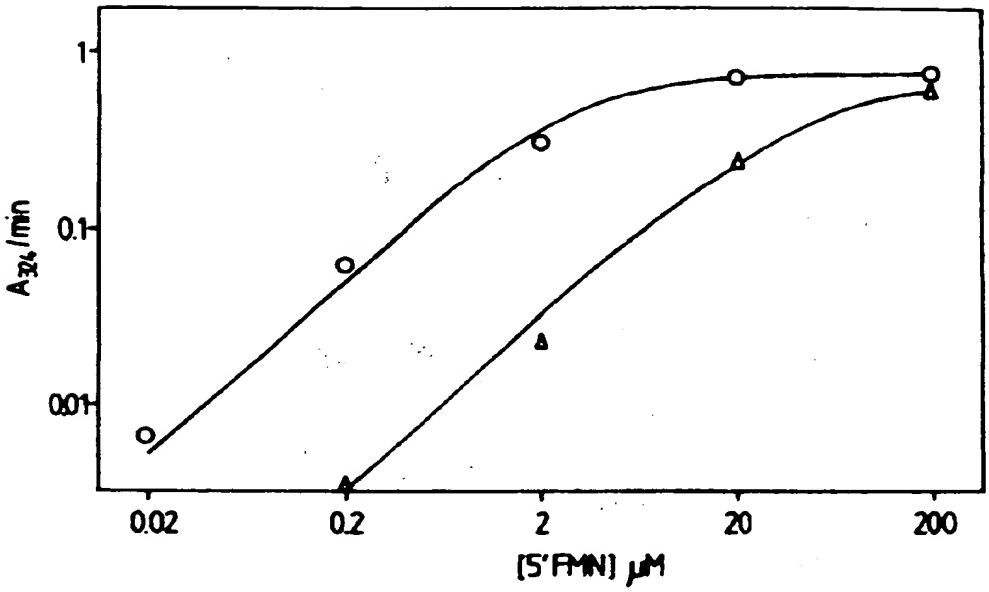

Amplification Assay
Absorbance at 492 nm
1
0.1
0.01
0.001
0.01
0.1
1
10
fmol Nuclease P1
pH 6.0
pH 6.3
pH 6.7

Figure 6



Sommerville & Rushton

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